Journal of Medical Virology 53:205–211 (1997)

Identification and Typing of Molluscum Contagiosum Virus in Clinical Specimens by Polymerase Chain Reaction

Carol H. Thompson*

Department of Infectious Diseases, Faculty of Medicine, The University of Sydney, NSW, Australia

A polymerase chain reaction (PCR) which enables the detection of molluscum contagiosum virus (MCV) genomes in either fresh or formalin-fixed clinical specimens is described. The primers used were designed to amplify a 167 bp region of the 3.8kbp *Hind*III fragment K of the MCV 1 genome. The ability of this PCR to detect three common MCV types (1, 1v and 2) in clinical specimens was confirmed using frozen extracts from 75 molluscum lesions, and digests of single sections of 11 formalin-fixed, paraffin-embedded lesions; all of which had been previously typed by Southern hybridisation. In addition, 2 specimens previously negative by hybridisation were shown to be positive for MCV DNA by PCR. Confirmation of the identity of the PCR products and distinction between the two major MCV types (MCV 1/1v versus MCV 2) was achieved by comparison of the results of cleavage with the restriction endonucleases *Hha*I and *Sac*I. Sequencing of the PCR products revealed complete homology between MCV 1 and 1v, but minor nucleotide variations between MCV 1/1v and MCV 2 were identified. As well as providing a highly sensitive means of diagnosis, the technique may also prove useful for investigations into the pathogenesis, epidemiology and natural history of molluscum contagiosum infection. ***J. Med. Virol. 53:205–211, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: molluscum contagiosum virus (MCV); poxvirus; polymerase chain reaction; DNA sequence

INTRODUCTION

Molluscum contagiosum virus (MCV) is a human poxvirus which produces benign tumour-like lesions of the skin which are characterised by hypertrophy and hypoplasia of the epidermis [Mehregan, 1961; Postlethwaite, 1970]. Infections in children and young adults are not uncommon, and in the latter group MCV most often occurs as a sexually transmitted disease [Brown, 1984; Oriel, 1987]. In individuals with normal immune function disease is usually self-limiting and is generally regarded as trivial. However, immunocompromised persons may be afflicted by severe MCV infections which are often refractory to the usual forms of therapy [Lombardo, 1985; Redfield et al., 1985; Katzman et al., 1987]. Since the infection cannot be transmitted to laboratory animals and a reliable in vitro system for MCV replication has yet to be established [Postlethwaite, 1970; McFadden et al., 1979], the epidemiology and pathogenesis of this disease remain largely unknown.

Restriction endonuclease analyses have demonstrated the heterogeneity of viral DNA obtained from clinical isolates [Parr et al., 1977; Darai et al., 1986]. The use of such analyses has enabled the establishment of a typing scheme used to investigate the molecular epidemiology of molluscum contagiosum infection. Three major genomic types with widespread distribution throughout the world have been identified: MCV 1 and its minor variant MCV 1v, MCV 2, and MCV 3 (previously "MCV 2v") [Darai et al., 1986; Porter and Archard, 1987; Scholz et al, 1988; Thompson et al., 1990; Porter and Archard, 1992]. In addition, there is evidence for the existence of at least one further type (MCV 4) and several other variants of MCV 1 (MCV 1vb and 1vc)—all of which may have geographically restricted distributions since they have not been reported outside Japan [Kawashima et al., 1990; Nakamura et al., 1995].

Various DNA hybridisation techniques have been used to identify the presence of MCV in clinical specimens [Thompson et al., 1990a, 1990b; Hurst et al.,

Contract grant sponsor: National Health and Medical Research Foundation.

*Correspondence to: Dr. C.H. Thompson, Department of Infectious Diseases, Blackburn Building (D06), The University of Sydney, NSW 2006, Australia. E-mail: carolt@med.su.oz.au

Accepted 17 June 1997

TABLE I. Summary of Clinical Data

No. of specimens	Sex of patients	Patients' HIV status	Mean age (range)	Lesion(s) location
29	Male	−ve	24.3 (2–47)	17: perigenital[a] 11: non-perigenital 1: multi-site
25	Female	−ve	21.5 (3–42)	11: perigenital 14: non-perigenital 0: multi-site
24	Male	+ve[b]	32.6 (21–56)	9: perigenital 12: non-perigenital 3: multi-site

[a]'Perigenital' includes lesions located on the penis, scrotum, labia, groin, perineum, perianal skin, medial upper thighs and pubis.
[b]Including 20 men with advanced symptomatic disease (AIDS or ARC), and 4 classified as PGL or asymptomatic.

1991; Forghani et al., 1992]. However, a more rapid and sensitive means of detecting the presence of viral DNA in clinical specimens would be a distinct advantage if the complex epidemiology of the disease is to be fully investigated, particularly in relation to the existence of latent or subclinical infections. In this report a polymerase chain reaction (PCR) technique, demonstrated to be capable of detecting very low amounts of viral DNA in either fresh and formalin-fixed specimens, is described.

MATERIALS AND METHODS

Typing of MCV DNA by Southern Hybridisation

Clinically diagnosed molluscum contagiosum lesions, consisting of curetted lesions or expressed lesion cores, were collected after informed consent had been obtained from 78 patients, 24 of whom were known to be HIV-positive, attending various STD and dermatology clinics in Sydney, Australia. Data regarding the gender, age and HIV status of these patients, together with the anatomical location of the lesions, are shown in Table I.

The specimens were frozen in liquid nitrogen until required. Nucleic acids were extracted from 1–10 mg of wet minced tissue by digestion in 600 μl lysis buffer, followed by standard phenol/chloroform extraction/ethanol precipitation and quantitation of total nucleic acids by spectrophotometric absorbance at 260 nm [Maniatis et al., 1982]. Detection and typing of MCV DNA was then carried out on 1–5 μg nucleic acids by restriction endonuclease cleavage with *Bam*HI, followed by high stringency Southern hybridisation with a digoxigenin-labelled MCV 1-F DNA probe, as previously described [Thompson et al., 1990]. In 11 of these 78 cases multiple closely-adjacent lesions had been submitted from the same patient. In these instances one of the lesions had been fixed in 10% neutral buffered formalin (NBF) for 18–48 hours, and histopathological examination on 5 μm H&E-stained paraffin sections had confirmed the presence of the pathognomonic features of molluscum contagiosum infection [Mehregan, 1961].

Polymerase Chain Reaction (PCR)

The entire sequence of the 190kb MCV 1 genome is now available (Genbank Accession No. MCU60315). However, when these investigations were commenced in 1995 only small regions had been sequenced [Blake et al, 1992; Bugert et al, 1993; Hadasch et al., 1993; Sonntag et al, 1995]. For the detection of MCV DNA by PCR, 23*mer* oligonucleotide primers amplifying a 167 bp (2171 to 2338 bp inclusive) region of the *Hind*III fragment K, encoding terminal repetitive sequences of the MCV 1 genome [Bugert et al., 1993; GenBank Accession No. M98814], were selected and synthesised (Gibco BRL Custom Primers). GenBank analyses revealed no known homology of these primers with either human or other viral DNAs. Primers GH20 and PC04,amplifying a 268 bp sequence of the human β-globin gene [Resnick et al., 1990], were also used in separate PCR reactions as controls for the integrity of the chromosomal DNA in each specimen tested. The sequences of both sets of primers are shown in Table II.

The remnants of the nucleic acid extracts used for Southern hybridisation had been stored at −20°C for periods of between 3 months to 6 years prior to amplification. In the case of the eleven paraffin-embedded specimens, crude lysates were prepared for PCR amplification. Briefly, single 5 μm sections were de-waxed by successive extractions in xylene and ethanol, desiccated, digested overnight at 37°C in 100 μl PCR lysis buffer (50 mM Tris HCl pH 8.5, 1 mM EDTA, 400 μg/ml Proteinase K, 0.5% Tween 20), held at 95°C for 8 min to inactivate the Proteinase K, then stored at −20°C (for up to 3 weeks prior to PCR).

In preliminary experiments optimal conditions for the amplification of the MCV DNA sequence were determined using varying concentrations of Mg^{2+} (from 1.0 to 3.0 mM), dNTPs (100 to 400 μM) and primers (0.25 to 2 μM) in chequerboard titrations with fixed amounts of target DNA in the PCR reaction mixtures. On the basis of these results (data not shown) the optimised PCR reaction mixtures (final volume of 25 μl for β-globin or 25μl/50 μl for MCV) contained 2.5 mM $MgCl_2$, 200 μM each dNTP (dCTP, dATP, dGTP, dTTP), 1 μM MCV primers or 0.5 μM β-globin primers, 0.5 U *Tth* polymerase (Biotech), 1× PCR reaction buffer (Biotech), and 5 μl (β-globin) or 5 μl/10 μl (MCV) of nucleic acid extract (diluted 1:200 in TE pH8) or undiluted paraffin section lysate. To assess the sensitivity of the assay, PCR was also carried out on twenty of the nucleic acid extracts diluted 10^{-3} to 10^{-7} in TE to give final approximate concentrations of total nucleic acids of 1 ng to 0.01 pg/μl; and on all 11 paraffin section lysates diluted from 10^{-1} to 10^{-4}. Sample processing negative controls included in each assay consisted of biopsies of neonatal foreskin or cervical carcinomas (extracts of frozen biopsy material or 5 μm paraffin sections), and reaction mixes without

TABLE II. Primers and Thermocycler Profiles Used for the Amplification of MCV DNA and β-globin DNA

Target	Primers and thermocycler profiles	Product (bp)
MCV	Primer1: $^{5'}$CCGATCTTTGCGAGCGTTCTTAA$^{3'}$ Primer 2: $^{5'}$TCCCATACAGCGAGGACAGCATA$^{3'}$ Thermocycler profile: 94°C 5 min (1 cycle) 94°C 30 sec, 54°C 1 min, 72°C 1 min (36 cycles) 94°C 30 sec, 54°C 1 min, 72°C 8 min (1 cycle)	167 (*Sac*I: 109, 58;[a] *Hha*I: 92, 43, 32)
β-globin	Primer 1: $^{5'}$GAAGAGCCAAGGACAGGTAC$^{3'}$ Primer 2: $^{5'}$CAACTTCATCCACGTTCACC$^{3'}$ Thermocycler profile: 94°C 5 min (1 cycle) 94°C 30 sec, 52°C 1 min, 72°C 1 min (40 cycles) 94°C 30 sec, 52°C 1 min, 72°C 8 min (1 cycle)	268

Expected sizes of the amplified MCV product, before and after cleavage with restriction endonucleases, are indicated.
[a]MCV 1 and 1v only; MCV 2 PCR products fail to cut with *Sac*I.

nucleic acids interspersed at 7-tube intervals. The possibility of inadvertent specimen-to-specimen and PCR product-to-specimen contamination was minimised by the use of dedicated equipment and by the routine application of protocols designed to minimise cross-contamination both before and after amplification.

Amplification was carried out in a thermal cycler (Corbett Research) set to profiles for specific amplification of target DNA as published previously for β-globin [Resnick et al., 1990], or as determined for MCV by preliminary experiments (Table II). Seven μl of each amplified product were electrophoresed through 2% Nu-Sieve agarose gels (150 V for 60 min), then stained with 1 μg/ml ethidium bromide and photographed under UV transillumination. The size of the amplicons was estimated by comparison with the bands produced by 0.25 μg pUC19DNA/*Hpa*II molecular size marker (Biotech).

Restriction endonuclease cleavage of the MCV PCR products was carried with the enzymes *Hha*I and *Sac*I, each with expected specific recognition sites within the region of DNA amplified (as indicated by the MacIntosh computer program *DNA Strider 1.0*). Ten μl of each PCR product was digested (37°C for 60 min) with 10 U of each enzyme under the conditions prescribed by the manufacturer (Promega), and the digests electrophoresed and examined as described above.

Sequencing of MCV PCR Products

MCV PCR products purified by PEG (polyethylene glycol) precipitation (Lis, 1980) were directly sequenced using the *Taq*DyeDeoxyTerminator cycle sequencing system (Applied Biosystems). Briefly, 45 μl PCR product were incubated with an equal volume of solution containing 26.7% PEG 800 (Sigma), 0.6M sodium acetate pH 5.2, 6.5 mM $MgCl_2$ for 60 min at 4°C. The DNA was pelleted by microfuging at 13 000rpm for 15 min, washed twice with 300 μl absolute alcohol, dried at 42°C, then rehydrated in 25 μl sterile water. The cycle sequencing reaction mix was then assembled using reagents from the *Taq*DyeDeoxy Terminator kit, 100–150 ng PEG-purified PCR product, 30 ng of forward or reverse PCR primers (in separate reactions) in a final volume of 20 μl. Cycle sequencing followed by removal of excess dye terminators by phenol/chloroform extraction and ethanol precipitation was undertaken essentially according to the manufacturer's instructions (Applied Biosystems). The purified extension products were then sequenced using an AB373A DNA Sequencer. Comparison of resultant sequences with those deposited in Genbank was carried out using *Blast* software.

RESULTS

All fresh tissue extracts (up to 1:200 dilution) and paraffin section digests (undiluted or 1:10 dilution) contained amplifiable human chromosomal DNA, as evidenced by the presence of appropriately sized bands (268 bp) after β-globin amplification (Fig. 1). However, the signals emanating from β-globin amplification rapidly diminished in the highly diluted samples, and in most instances negative results were obtained from those samples containing less than 10 pg total extracted nucleic acids, or in paraffin section lysates diluted more than 1:10. Amplification of MCV DNA resulted in single bands of expected size (~70 bp) from 77 of the 78 nucleic acid extracts and from all eleven paraffin section lysates (Fig. 1). None of the negative controls produced bands of any size. The MCV assay was also able to produce readily visualised amplicons from the all 20 specimen extracts diluted to contain an estimated 1 ng to 0.1 pg total nucleic acids; and in the majority of instances (17/20) clear signals were also obtained with the 10^{-7} dilutions, representing as little as 0.01 pg of specimen nucleic acids as target (Fig. 2). All of the formalin-fixed paraffin section of molluscum lesions produced clear evidence of the presence of MCV

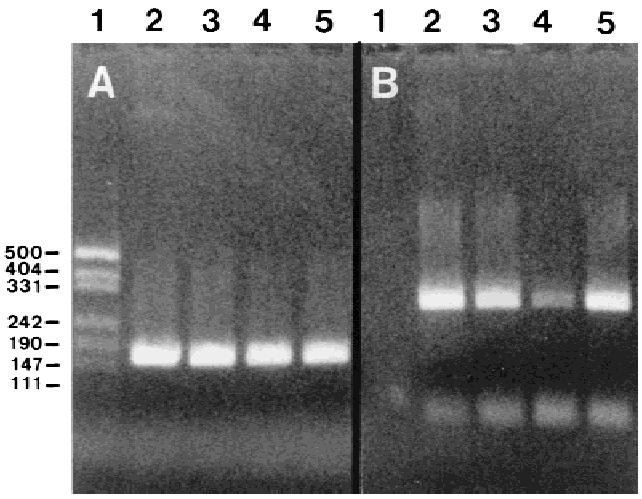

Fig. 1. Result of PCR amplification for MCV (Panel A) and β-globin (Panel B) on molluscum lesion extracts. **Panel A, Lane 1:** pUC19/*Hpa*II digest size markers. **Panel A, Lanes 2–5:** 167 bp bands indicative of MCV DNA amplified from four unfixed lesion extracts (diluted 1:1000 in TE buffer). **Panel B, Lane 1:** Negative control (lysis buffer). **Panel B**, **Lanes 2–5:** 268bp amplicons resulting from β-globin PCR on extracts (diluted 1:200) of the same four specimens shown in Panel A.

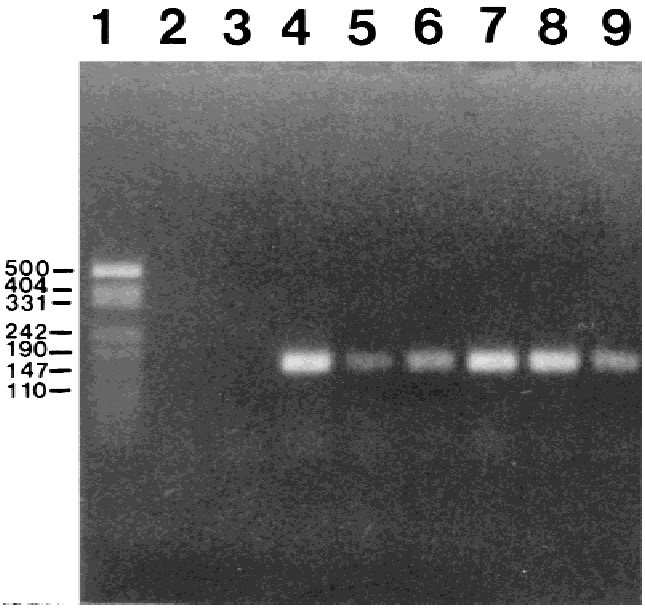

Fig. 2. Example of sensitivity of the MCV PCR protocol on two of the unfixed molluscum lesion extracts. **Lane 1:** pUC19/*Hpa*II digest size markers. **Lanes 2 and 3:** negative controls (lysis buffer and water). **Lanes 4–6:** specimen MCV130 diluted 10^{-5}, 10^{-6}, 10^{-7} in TE buffer. **Lanes 7–9:** specimen MCV 135diluted 10^{-5}, 10^{-6}, 10^{-7} in TE buffer.

DNA up to and including the 1:100 dilution (8 specimens) or 1:1000 dilution (3 specimens) of section lysate.

Previous hybridisation experiments had indicated the presence of MCV DNA in 75 of the 78 specimens examined by PCR in the current investigation. Two of the specimens considered previously negative for MCV DNA by Southern hybridisation were found to be positive by PCR; while remaining specimen was negative

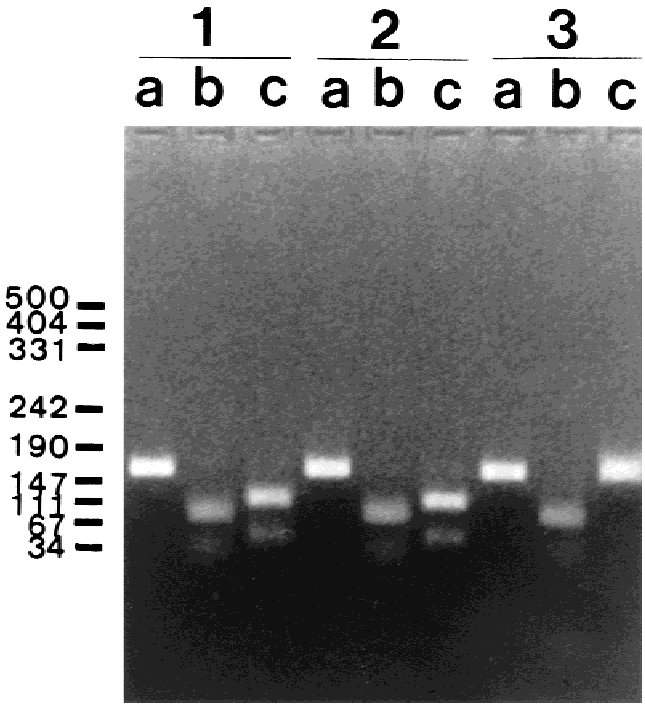

Fig. 3. Typing of MCV PCR products by restriction endonuclease digestion. **Panel 1, 2 and 3:** MCV type 1, type 1v and type 2 PCR products, respectively. The products were uncut (**Lanes 1a, 2a, 3a**); digested with *Hha*I (**Lanes 1b, 2b, 3b**); or *Sac*I (**Lanes 1c, 2c, 3c**). The smaller fragments resulting from *Hha*I cleavage (43 and 33 bp) are running too close together on this gel to be distinguished.

for MCV DNA by both Southern hybridisation and PCR. Typing of the nucleic acid extracts by *BamH*I cleavage followed by Southern hybridisation with a digoxigenin-labelled MCV 1-F probe (Thompson et al., 1990) had revealed that 44 contained MCV 1 or its minor variant MCV 1v, while 31 were positive for MCV 2. Discrimination between these two major MCV types was found to be possible by cleavage of the PCR products with *Hha*I and *Sac*I: *Hha*I digested MCV type 1, its minor variant MCV 1v, and MCV 2 products identically producing 3 fragments of 92, 43 and 33 bp; whereas *SacI* sucessfully cleaved MCV 1 and 1v (fragments of 109 and 58 bp), but failed to cut the PCR product resulting from MCV 2 amplification (Fig. 3). In 74 cases cleavage of the PCR product produced a result consistent with those of Southern hybridisation (43 MCV 1 or 1v; 31 MCV 2); but the product from one specimen consistently failed to completely digest with *Sac*I on repeat amplifications, indicating the possibility of a simultaneous infection with both MCV 1 and MCV 2. Two of the three specimens previously negative for MCV DNA were shown by PCR to be positive for MCV 1/1v. When the PCR products from the paraffin section lysates were digested with *Hha*I and *Sac*I cleavage the results were in agreement with the Southern hybridisation typing results on adjacent lesions (9 lesions type 1 or 1v; 2 lesions type 2).

Forward and reverse sequencing of PCR products was carried out on six specimens: two each of MCV 1,

TABLE III. Sequencing Results of MCV Types 1, 1v and 2 PCR Products Compared with the nt 2171–2338 (M98814) and nt 5209–5375 (U60315) Sequences of MCV 1 in GenBank

	2171/5209	*Hha*I
M98814	CGGATCTTTG CGAGCGTTCT TAATGTTTTC CTCGCAGTAG	CGGCGCTCCT
U60315	CGGATCTTTG CGAGCGTTCT TAATGTTTTC CTCGCAGTAG	CGGCGCTCCT
PCR MCV 1	*CGGATCTTTG CGAGCGTTCT TAA*TGTTTTC CTCGCAGTAG	CGGCGCTCCT
PCR MCV 1v	*CGGATCTTTG CGAGCGTTCT TAA*TGTTTTC CTCGCAGTAG	CGGCGCTCCT
PCR MCV 2	*CGGATCTTTG CGAGCGTTCT TAA*TGTTTTC CTCGCAGTAG	CGGCGCTCCT
	2221/5259 *Hha*I	
M98814	CTGCGATCAT CGACATGTAG GCAGT	GCGCT GTGTTTGCCA CCGTTGGTAA
U60315	CTGCGATCAT CGACATGTAG GCAGT	GCGCT GTGTTTGCCA CCGTTGGTAA
PCR MCV 1	CTGCGATCAT CGACATGTAG GCAGT	GCGCT GTGTTTGCCA CCGTTGGTAA
PCR MCV 1v	CTGCGATCAT CGACATGTAG GCAGT	GCGCT GTGTTTGCCA CCGTTGGTAA
PCR MCV 2	CTGCGATCAT CGACATGTAG GC-GT	GCGCT GTGTTTGCCA CCGTTGGTAA
	2271/5309 *Sac*I	2311/5348
M98814	ACACTGCAA	GA GCTCAAGTGA GGTCTTTCGA GAA**A**CACGCA C**GCG**GTATGC
U60315	ACACTGCAA	GA GCTCAAGTGA GGTCTTTCGA GAA-CACGCA C**AGC**GTATGC
PCR MCV 1	ACACTGCAA	GA GCTCAAGTGA GGTCTTTCGA GAA-CACGCA C**AGC**GT*ATGC*
PCR MCV 1v	ACACTGCAA	GA GCTCAAGTGA GGTCTTTCGA GAA-CACGCA C**AGC**GT*ATGC*
PCR MCV 2	ACACTGCAA	GA G**T**TCAAGTGA GGTCTTTCGA GAA-CACGCA C**AGC**GT*ATGC*
	2321/5358	
M98814	TGTCCTCGCT GTATGGGA 2339	
U60315	TGTCCTCGCT GTATGGGA 5375	
PCR MCV 1	*TGTCCTCGCT GTATGGGA*	
PCR MCV 1v	*TGTCCTCGCT GTATGGGA*	
PCR MCV 2	*TGTCCTCGCT GTATGGGA*	

Primers are indicated by italics, and the expected cleavage sites for *Hha*I and *Sac*I are underlined. Nucleotides found to be at variance with published sequences are shown in bold.

MCV 1v and MCV 2. The results for each set were identical, and are shown in Table III, together with the sequence of the 2171–2340 region of MCV 1 published by Bugert et al. [1993] and the entire MCV 1 sequence deposited in Genbank. The sequences of the 167 bp MCV 1 and MCV 1v PCR products were found to be homologous, and were identical with the 5209–5375 bp region of the entire MCV genome. However, they differed from the 2171–2339 bp sequence of Bugert et al. by the deletion of A at nt 2304/5342, and the replacement of AGC for GCG at nts 2312–2314/5349–5351. No sequencing data for MCV 2 was available from Genbank; however, in this study the type 2 PCR product showed variations from those of types 1 and 1v: A was deleted at nt 2243/5281; and T replaced C at nt 2282/5320 (resulting in the loss of the *Sac*I cleavage site).

DISCUSSION

The use of PCR to identify and type MCV infection has been reported previously [Bugert and Darai, 1995; Nunez et al., 1996]. However, these workers only described its use in extracts of fresh clinical samples and provided no estimation of its level of sensitivity. The protocol in this report was found to provide a convenient and extremely sensitive means for the detection and typing of the viral DNA in various types of clinical specimens, including formalin-fixed paraffin sections. Although one of the submitted lesions did not produce evidence of the presence of MCV DNA, this may not necessarily indicate a shortcoming in the technique since it is well recognised that errors in the clinical identification of molluscum contagiosum may occur. In this case histopathological confirmation had not been attempted, and it is likely that the lesion was not in fact caused by MCV. In a diagnostic setting the technique would permit the identification of MCV in fresh specimens and in cell cultures, even in those instances where minimal amounts of clinical material are provided, and would be particularly useful in retrospective surveys when only fixed material is available.

Currently there is only limited information on the molecular epidemiology of molluscum contagiosum, although it would appear that the relative occurrence of the various genomic types varies greatly in different regions of the world. In Australia the percentage of infections caused by MCV 2 (25%) is more similar to that seen in England (19%, Porter and Archard, 1987) than in either Germany (3.4%, Scholz et al, 1988), Japan (2.7%, Nakamura et al., 1995) or Spain (2.6% Nunez et al., 1996). The interpretation of these figures should, however, be made with caution, given the fact that it has previously been shown that the ratio of MCV 2 to MCV 1/1v is much greater in adults as compared with children, and is higher in perigenital lesions as compared with those located on other parts of the body [Thompson et al., 1990; Thompson et al., 1992]. The source of lesion material may therefore significantly bias the typing ratios in one or other direction. In this survey a substantial proportion of the samples investigated (37/78, 47%) had been collected from adults with perigenital lesions, presumably representing disease transmitted by sexual contact. This may have been a significant factor contributing to the high percentage of lesions found to be positive for MCV 2.

Infections due to MCV 3 appear to be universally uncommon. It would have been interesting to include a specimen positive for MCV 3 in the PCR assay, but

unfortunately due to its rare occurrence no material positive for this type was available at the time of these investigations.

The incidence of MCV infection in the general community remains uncertain. It is difficult for valid figures to be compiled since molluscum contagiosum is not a notifiable disease in any country, and the generally trivial nature of the infections means that in many instances persons with molluscum lesions will not seek medical attention. Nonetheless, a survey of the medical records of Dutch general practitioners found that the infection is quite common in children, with a cumulative incidence of 17% in those aged under 15 years and under [Koning et al., 1994]. These workers considered that the adult sexually transmitted form of the disease was rare; but recent data from New Zealand STD clinics has shown that in 1995 0.7% of the 30,301 attendees were diagnosed with molluscum contagiosum [Lyttle and Preston, 1997]. This figure compared with 8.2% and 3.7% for the most common viral STDs (anogenital warts and anogenital herpes infections, respectively); and 2.5%, 0.5% and 0.03% for the bacterial infections chlamydia, gonorrhoea and syphilis. As in most other regions of the western world the decline in bacterial STDs over past decades has been accompanied by a relative and absolute increase in the number of viral infections.

Some workers have suggested that MCV, like certain other DNA viruses, may exist in a latent form [Katzman et al., 1987], and it is conceivable that in many instances subclinical infections may occur. The latter hypothesis has been strengthened by the results of a recent investigation using an ELISA to detect IgG antibodies to MCV type 1, type 1v or type 2 purified virion proteins in adults with no known history of molluscum infection [Konya et al., 1992]. Almost one-third of those surveyed demonstrated serological evidence of past exposure to the virus,indicating that mild or subclinical infections may be much more common than previously suspected. The use of sensitive PCR technology to identify and type MCV DNA, such as those decribed in this report and the previous paper of Nunez et al. [1996], may therefore prove to be valuable in clarifying the epidemiology and pathogenesis of this interesting but enigmatic viral infection. The ability to detect very low levels of virus in clinical specimens more readily obtained than the usual biopsies or lesion cores (such as scrapings or swabs), in paraffin sections, or in environmental samples may also be a significant advantage in elucidating the natural history of the disease.

ACKNOWLEDGMENTS

This study was partly funded by a grant from The National Health and Medical Research Foundation.

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